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Stimulation by Adenosine 3′,5′-Cyclic Monophosphate of Protein Synthesis by Adenohypophyseal Polyribosomes*

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ABSTRACT: Addition of dibutyryl 3′,5′-cyclic AMP to slices of bovine pituitary stimulated incorporation of [^{3}H]leucine into protein, whether or not actinomycin D was present; therefore the influence of 3′,5′-cyclic AMP on protein synthesis by bovine pituitary polysomes was studied. If the cyclic nucleotide was added to the complete protein-synthesizing system (including pH 5.0 enzyme), stimulation of [^{3}H]leucine incorporation occurred only with pH 5.0 enzyme from rat liver; there was no stimulation when homologous enzyme, *i.e.*, from bovine pituitary, was used. Addition of 3′,5′-cyclic AMP to the polysomes, before addition of pH 5.0 enzyme, resulted in stimulation of protein synthesis with either source of enzyme, but stimulation was facilitated to a greater degree, over the range 0.5–2 mM 3′,5′-cyclic AMP, when rat liver was the source. The stimulation of protein synthesis was prevented by the addition of cycloheximide. With rat liver pH 5.0 enzyme the product of hydrolysis of 3′,5′-cyclic AMP was mainly 5′-AMP whereas with pituitary pH 5.0 enzyme there was also dephosphorylation and deamination resulting in production of hypoxanthine and other bases. However, using either source of pH 5.0 enzyme and the complete protein-synthesizing system (*i.e.*, including an ATP-regenerating mechanism) most of the ^{3}H from hydrolysis of [^{3}H]3′,5′-cyclic AMP was incorporated into ATP. The data are seen as compatible with a stimulation by 3′,5′-cyclic AMP of translation by pituitary polysomes; the significance of the importance of the source of pH 5.0 enzyme used in the system is obscure.

The mediation of many hormonal effects on target tissues by 3′,5′-cyclic AMP has been increasingly recognized in recent years (Robison *et al.*, 1968). The great majority of the effects are characterized as rapid "purely metabolic" (Sutherland *et al.*, 1967) actions, such as increase in the rate of glycogenolysis as an effect of epinephrine on liver (Sutherland and Robison, 1966) or release of stored hormone, exemplified by release of thyroid hormone by thyrotropin (Bastomsky and McKenzie, 1967) or of thyrotropin by thyrotropin-releasing factor (Wilber *et al.*, 1969). However, there is some evidence that 3′,5′-cyclic AMP may mediate other hormonal effects involving biosynthetic mechanisms, such as thyrotropin-enhanced phospholipid synthesis in the thyroid (Pastan and Macchia, 1967), and we have observed stimulatory effects of dibutyryl 3′,5′-cyclic AMP *in vitro* on RNA and protein incorporation of labeled precursors in thyroid (Adiga *et al.*, 1971) and pituitary slices (McKenzie *et al.*, 1970). In investigating the mechanism of the phenomenon we recognized a stimulation of RNA polymerase activities of subsequently isolated nuclei when dibutyryl 3′,5′-cyclic AMP was added to pituitary slices (McKenzie *et al.*, 1970). However, as reported here, the stimulation by dibutyryl 3′,5′-cyclic AMP of [^{3}H]leucine incorporation into protein in pituitary slices still occurred to some extent in the presence of actinomycin D. Consequently we turned to study the influence of 3′,5′-cyclic AMP on protein synthesis in a bovine pituitary cell-free system (Adiga *et al.*, 1966, 1968) that did not involve RNA synthesis and hence was not affected by actinomycin D.

Experimental Section

Materials. L-[^{3}H]Leucine, 5 Ci/mmole, was purchased from New England Nuclear Corp. Pyruvate kinase, phosphoenol pyruvate (trisodium salt), and 3′,5′-cyclic AMP were obtained from Sigma Chemical Co., dibutyryl 3′,5′-cyclic AMP and [^{3}H]3′,5′-cyclic AMP, 1.4 Ci/mmole, from Schwarz BioResearch, and [^{32}P]3′,5′-cyclic AMP, 6.1 Ci/mmole, from International Chemical & Nuclear. Nonradioactive AMP, ADP, ATP, adenine, adenosine, xanthine, and inosine were all from Nutritional Biochemical Corp. The source of other biochemicals used was previously described (Adiga *et al.*, 1966, 1968).

Preparation of the Constituents of the Anterior Pituitary Cell-Free System. Fresh bovine pituitary glands were obtained from a local abatoir. The processing of the glands and the methods of preparing a polysome-enriched suspension, pH 5.0 enzyme and supernatant fractions, and reaction mixture that contained energy sources, [^{12}C]amino acid mixture, and cofactors were previously described in detail (Adiga *et al.*, 1966, 1968). Rat liver pH 5.0 enzyme and super-

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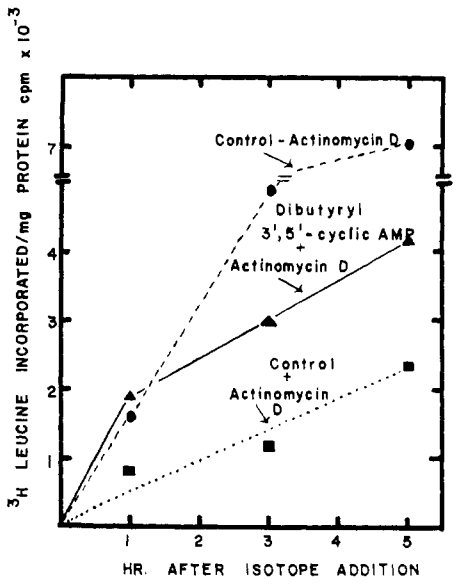

FIGURE 1: [^{3}H]Leucine incorporation into protein in bovine anterior pituitary slices: effect of dibutyryl 3',5'-cyclic AMP in the presence of actinomycin D. Tissue slices (150 mg) were incubated at 37° with or without actinomycin D, 50 μg per ml of medium, and 1 mM dibutyryl 3',5'-cyclic AMP. Incubations were in 4 ml of Krebs–Ringer bicarbonate buffer, pH 7.5, supplemented with medium 199 (10% v/v). After 1 hr of preincubation 8 μCi of [^{3}H]leucine was added and at the specified times aliquots of tissue were removed, labeled protein was isolated, and specific activity determined, as detailed previously (Adiga *et al.*, 1966). Similar data were obtained in a duplicate experiment.

natant fractions were prepared in similar fashion using young adult rats. The complete cell-free system contained pituitary polysomes (approximately 100 μg of rRNA), 0.5 mg of each of pH 5.0 enzyme and pH 5.0 supernatant factors, from either pituitary or liver tissue, and the other constituents referred to above, together with 2 μCi of ^{3}H-labeled precursor in a total volume of 0.25 ml. The polysome and enzyme preparations were used either fresh or after storage at −20°. Incubations of the reaction mixture were at 37°.

Assay of Cyclic Nucleotide Phosphodiesterase Activity in Preparations of pH 5.0 Enzyme and Supernatant Factor and Analysis of the Products of Metabolism of [^{3}H]3',5'-Cyclic AMP Added to pH 5.0 Enzyme Preparations or the Complete Cell-Free System. To estimate phosphodiesterase activity 0.1 ml of 1.0 mM 3',5'-cyclic AMP containing 0.5 μCi of [^{3}H]3',5'-cyclic AMP, in 0.05 M Tris-HCl, pH 7.4, with or without $MgSO_4$, was added to 0.1 ml of solution of pH 5.0 enzyme or supernatant factor and, after mixing, they were incubated, with shaking, at 37° for 3 to 15 min; the reaction was terminated by placing the tube containing the mixture in boiling water for 3 min. Duplicate aliquots (20–30 μl) of the mixtures were transferred to filter paper for ascending chromatography. Chromatography systems used were: A, 95% ethanol–1.1 M ammonium acetate, 74:26; B, 1-propanol–ammonia–water, 60:30:10; C, distilled water; D, 1-butanol–ammonia, 86:14; E, isobutyric acid–2.0 N ammonia, 66:34. Variably, 0.01 ml of approximately 1 mM solutions of nonradioactive nucleotides, nucleosides, and bases (3',5'-cyclic AMP, ATP, ADP, AMP, adenosine, hypoxanthine, xanthine, inosine, and adenine) was run in parallel or together with the test solutions and their position on the finally dried chromatograms was identified by use of ultraviolet light. Appropriate portions of the chromatograms were cut out and placed in vials containing 2 ml of toluene with 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter; ^{3}H was counted in a Packard liquid scintillation spectrometer. The percentage of [^{3}H]-3',5'-cyclic AMP remaining was calculated, and where indicated, the proportion of radioactivity appearing in metabolites also was determined. Corrections for nonspecific degradation of substrate were made by concomitant analysis of [^{3}H]3',5'-cyclic AMP incubated with boiled enzyme preparations; hydrolysis in these control tubes was never more than 2%. In two experiments 2 μCi of [^{32}P]3',5'-cyclic AMP was included in the reaction mixture and the distribution of both ^{3}H and ^{32}P established on subsequent chromatographic analysis.

Results

As indicated in the introduction 1 mM dibutyryl 3',5'-cyclic AMP stimulated precursor incorporation into protein in bovine pituitary slices *in vitro;* there was, for instance, 110% increase in incorporation of [^{3}H]leucine over 180 min of incubation (McKenzie *et al.*, 1970). Figure 1 shows that such stimulation occurred in the presence of actinomycin D, 50 μg/ml of medium; despite over 50% inhibition of [^{3}H]leucine incorporation into protein by preincubation with actinomycin D, incorporation was enhanced more than twofold by dibutyryl cyclic AMP within 1 hr of addition of the [^{3}H]leucine. Despite this indication that stimulation of protein synthesis could occur apart from stimulation of RNA synthesis, the isolated pituitary polysome preparation, using pituitary as the source for pH 5.0 enzyme, was not stimulated by 1 mM 3',5'-cyclic AMP in its ability to incorporate [^{3}H]leucine, whether or not 1 mM theophylline, an inhibitor of 3',5'-cyclic AMP phosphodiesterase (Butcher and Sutherland, 1962), was present (Figure 2A). When rat liver was taken as the source of pH 5.0 enzyme, incorporation of [^{3}H]leucine by the pituitary polysomes was enhanced (approximately 50% in 30 min) by the addition of 1 mM 3',5'-cyclic AMP (Figure 2B). The addition of 1 mM theophylline not only did not enhance this effect but actually decreased the stimulation (by about 27% in 30 min of incubation); 1 mM AMP slightly inhibited incorporation. An identical pattern of results was obtained with 3 different preparations of polysomes and enzymes.

Preincubation of 3',5'-cyclic AMP with either of the pH 5.0 enzyme preparations before addition of pituitary polysomes prevented in both cases stimulation of [^{3}H]leucine incorporation. However, the stimulatory effect on the incorporation of [^{3}H]leucine was evident if the pituitary polysomes were preincubated with the cyclic nucleotide, regardless of whether it was pituitary or liver pH 5.0 enzyme that was subsequently used in the reaction mixture. Therefore, by means of preincubating polysomes with 3',5'-cyclic AMP, the dose–response relationship was established and, as detailed in Figure 3, there was stimulation of [^{3}H]leucine incorporation directly related to the concentration of nucleotide (over 0.5–2.0 mM) when liver pH 5.0 enzyme was used; with pituitary pH 5.0 enzyme stimulation was observed with 1.0 mM 3',5'-cyclic AMP and a greater concentration of nucleotide was no more effective. For either source of pH 5.0 enzyme, 0.5–2.0 mM dibutyryl 3',5'-cyclic AMP was inhibitory. Again, similar data were obtained with 3 different preparations of liver and pituitary enzymes. Preincubation of the total protein-synthesizing system for 10 min did not diminish its efficiency in incorporating [^{3}H]leucine but

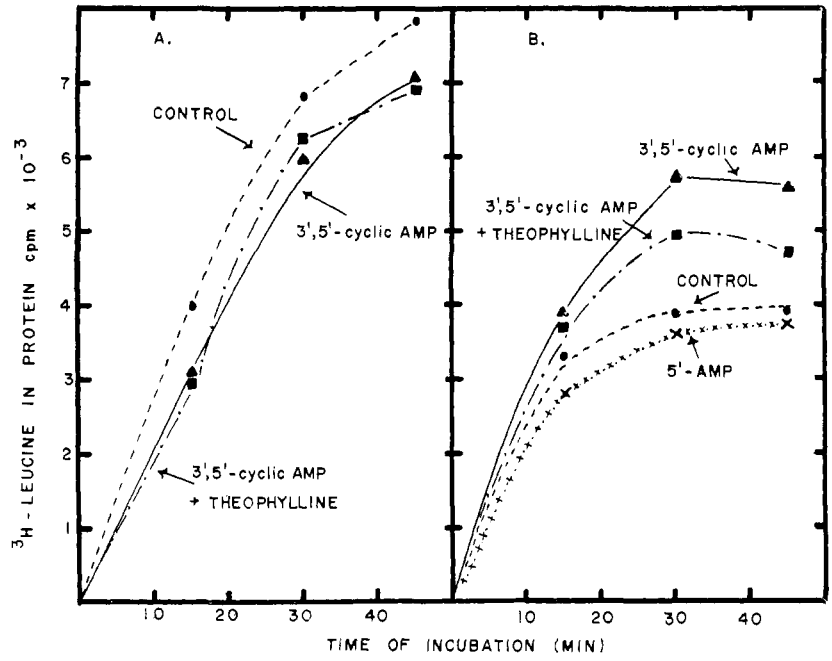

FIGURE 2: Influence of the source of pH 5.0 enzyme on the time course of incorporation of [^3H]leucine into protein by anterior pituitary polysomes and the effects of 1 mM 3′,5′-AMP, 1 mM 5′-AMP, and 1 mM theophylline. Pituitary polysome preparations (approximately 100 μg of rRNA) were incubated with either (A) pituitary or (B) rat liver pH 5.0 enzyme and supernatant factor, 1 mg of protein in each instance, and nucleotides (± theophylline) in the complete reaction mixture; total volume 0.25 ml. Assays were performed in duplicate. Other details are given in the text.

addition of 3′,5′-cyclic AMP after such preincubation led to less consistent stimulation of incorporation.

In attempts to explain the difference, related to source of pH 5.0 enzyme, in the effect of 3′,5′-cyclic AMP, we analyzed by paper chromatography the *in vitro* metabolism of 1 mM [^{3}H]3′,5′-cyclic AMP by the two enzyme preparations. Both enzyme preparations (1 mg of enzyme protein) hydrolyzed the nucleotide by over 80% in 10-min incubation but the products differed. In the five solvent systems (see Methods) the major product of hydrolysis of 3′,5′-cyclic AMP by pituitary pH 5.0 enzyme had an R_F similar to that of hypoxanthine; the product of hydrolysis by liver pH 5.0 enzyme ran as 5′-AMP in each system. Confirmation of dephosphorylation was obtained by analyzing the products of hydrolysis by pituitary pH 5.0 enzyme of 1 mM 3′,5′-cyclic AMP doubly labeled with ^{3}H and ^{32}P. In 10 min at 37°, 1 mg of enzyme protein caused 45% hydrolysis; 37% of the ^{3}H label but only 0.1% of ^{32}P traveled in the hypoxanthine area on chromatography (solvent system B) whereas 35% of ^{32}P and 1.4% ^{3}H occurred in the phosphate area. However, addition of [^{3}H]3′,5′-cyclic AMP to the total reaction mixture, including polysomes and pH 5.0 enzyme of either source, resulted in partial hydrolysis of the nucleotide (32% by the mixture that included pituitary pH 5.0 enzyme and 44% by the other) and the major product in both instances was chromatographically indistinguishable from ATP.

In the presence of 10 mM theophylline the hydrolysis of 1 mM [^{3}H]3′,5′-cyclic AMP by pH 5.0 enzyme of either source was reduced to about 5% per mg of enzyme protein in 10 min at 37°.

As shown in Figure 4, stimulation of incorporation of [^{3}H]leucine by pituitary polysomes was very sensitive to inhibition by cycloheximide, being prevented by 20 μg/ml, the smallest dose tested; larger concentrations of cycloheximide, to 200 μg/ml, were associated with inhibition of [^{3}H]-leucine incorporation that was greater (by up to 20%) in tubes containing 1 mM 3′,5′-cyclic AMP.

Discussion

There are now several lines of evidence that 3′,5′-cyclic AMP can stimulate protein synthesis *in vitro*. Pastan and Perlman (1969) reported increased synthesis of tryptophanase in a preparation of *Escherichia coli* with 0.4 mM 3′,5′-cyclic AMP; Chambers and Zubay (1969) found that 1 mM 3′,5′-cyclic AMP added to a bacterial cell-free system improved the yield of β-galactosidase enzymatic activity. In neither of these instances was there enhancement of overall protein synthesis. However, Grand and Gross (1969) more recently reported almost doubling of *in vitro* incorporation of [^{14}C]L-amino acids into protein by slices of rat parotid gland as an effect of 2 mM dibutyryl 3′,5′-cyclic AMP. Langan (1968) described an enhanced rate of phosphorylation of histone, in a system catalyzed by a liver enzyme preparation, when 3′,5′-cyclic AMP was present at 10^{-7} M concentration, and suggested that this might be the basis of induction of RNA synthesis by hormones that increase a tissue's concentration of 3′,5′-cyclic AMP. In studies with bovine pituitary and porcine thyroid slices we found stimulation of incorporation of [^{3}H]leucine into protein and [^{3}H]uridine into RNA on incubation with 1 mM dibutyryl 3′,5′-cyclic AMP; RNA polymerase activities of nuclei subsequently isolated from such slices were enhanced (McKenzie *et al.*, 1970). While the last finding, together with the other reports just described, leads to speculation that an effect of 3′,5′-cyclic AMP on protein synthesis might center on activation of RNA polymerase, our finding that the stimulatory effect of the dibutyryl ester on [^{3}H]leucine incorporation was partly resistant to actinomycin D in a dose capable of 80–85% inhibition of RNA synthesis *in vitro* (unpublished data) indicated that

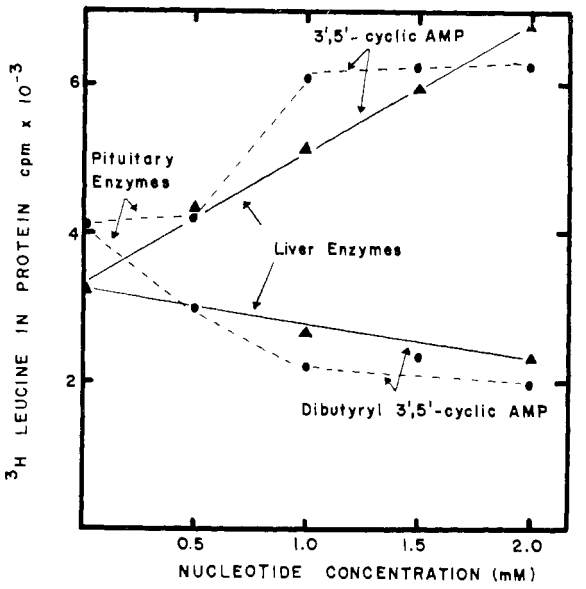

FIGURE 3: Effect of preincubation of pituitary polysomes with 3',5'-cyclic AMP or dibutyryl 3',5'-cyclic AMP on [^{3}H]leucine incorporation into protein: influence of source of pH 5.0 enzyme on dose–response relationships. Polysomes were preincubated for 10 min at 37° with specified amounts of the nucleotides and other necessary constituents; pH 5.0 enzyme and supernatant factor and [^{3}H]leucine were then added and incubations were continued for 30 min. Labeled protein was then isolated and radioactivity determined. Other details were as given in Figure 2.

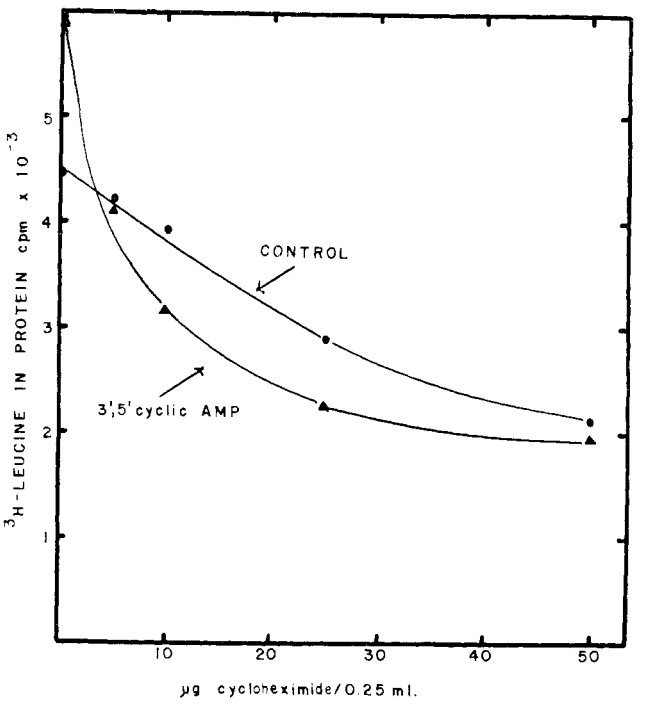

FIGURE 4: The effect of increasing concentration of cycloheximide on 3',5'-cyclic AMP-stimulated incorporation of [^{3}H]leucine into protein by pituitary polysomes. Incubations were for 10 min at 37° before addition of rat liver pH 5.0 enzyme (1 mg of protein per tube) and supernatant factor, thus establishing the complete system in a volume of 0.25 ml; incubations were continued for a further 30 min. Other details are given in Figure 2.

an additional site must be considered. Furthermore, while this work was in progress, Lissitzky *et al.* (1969) reported that preincubation with 3',5'-cyclic AMP (0.2 and 0.4 mg/ml) stimulated incorporation of [^{14}C]leucine by thyroid polyribosomes (and, in passing reference, by a rat liver cell-free ribosome system); they considered this was unspecific stimulation, in part because poly(U)-directed polyphenylalanine synthesis, by a thyroid cell-free ribosome system, also was stimulated. Similar stimulation of polyphenylalanine formation was reported also using reticulocyte ribosomes (Malkin and Lipmann, 1969). Since poly(U)-directed polyphenylalanine synthesis does not require initiation factor (Eisenstadt and Brawerman, 1967) this might indicate that the action of 3',5'-cyclic AMP is beyond the step of chain initiation.

Addition of 3',5'-cyclic AMP to the complete protein-synthesizing system containing pituitary pH 5.0 enzyme was without effect. That this failure might be due to the activity of cyclic nucleotide phosphodiesterase (shown to be in the pH 5.0 enzyme preparation) was supported by the facts that 3',5'-cyclic AMP was stimulatory when added to the polysome system before the enzyme and that there was no stimulation by 5'-AMP, the product of hydrolysis of 3',5'-cyclic AMP by phosphodiesterase. However this simple explanation failed to accommodate the finding that 3',5'-cyclic AMP stimulated protein synthesis by the complete polysome system when rat liver pH 5.0 enzyme was used, since this enzyme preparation also contained cyclic nucleotide phosphodiesterase.

We were unable to explain the specific importance of the source of pH 5.0 enzyme. While the products of hydrolysis of [^{3}H]3',5'-cyclic AMP by the enzyme differed markedly depending on the source, the pituitary enzyme preparation apparently causing deamination and dephosphorylation of 5'-AMP, this did not occur when the total reaction mixture was used as incubation medium; presumably the ATP-regenerating system in the mixture rapidly metabolized to ATP the AMP formed from 3',5'-cyclic AMP. Furthermore, there was no stimulation of [^{3}H]leucine incorporation by the pituitary polysomes when the cyclic nucleotide was incubated with pH 5.0 enzyme of either source before addition of the polysomes.

The lack of enhancement of the effect of 3',5'-cyclic AMP, and indeed the reduction of the effect, by 1 mM theophylline, despite adequate maintenance by the methylxanthine of the nucleotide in unhydrolyzed form, may be explained by the inhibition of [^{3}H]leucine incorporation that occurred with addition to the reaction mixture of theophylline alone (unpublished data). This finding is reminiscent of inhibition of adrenal protein synthesis by theophylline (Halkerston *et al.*, 1966) and our own findings of inhibition by theophylline of protein synthesis in slices of bovine pituitary and porcine thyroid (unpublished data).

The precise site of action of 3',5'-cyclic AMP in the polysome system is not clear. An action on translation would be compatible with the inhibition by a small dose of cycloheximide of stimulation of [^{3}H]leucine incorporation. While an effect at any other point in the sequence of protein synthesis would also be inhibited by cycloheximide, our finding that the stimulatory effect was resistant to actinomycin D and the reports, already noted, of stimulation by 3',5'-cyclic AMP of polymerization of an activated amino acid in other polysome systems indicate an influence on translational control.

It is noteworthy that the effects of 3',5'-cyclic AMP that we report as well as those referred to above, described by others, require concentrations of the nucleotide perhaps 1000-fold greater than have been measured in similar tissues.

Possible explanations include, for instance, that *in vivo* pools of the cyclic nucleotide may exist to establish increased concentrations at critical intracellular loci; alternatively a receptor or binding protein (Gill and Garren, 1970) may not be maintained appropriately in the cell-free systems used to provide optimal conditions for the effects studied. However, as long as there is no satisfactory explanation for the discrepancy, one may not discard the implication that changes requiring pharmacological dosages may not reflect physiological phenomena.

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